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DNA was precipitated with ethanol. The fragments were then separated on a 1.0% agarose gel, using the λ DNA as a control for the performance of the restriction enzymes. Human genomic DNA from the same cell line was also digested using the enzymes Eco RI and Hind 3 as described above. The size of the restriction fragments that contain the target sequence of probe 1 in DNA digested with Bgl 2 Eco Rl and Hind 3 was 5803, 1570 and 3390 base pairs, respectively.

For analysis with probes 5 and 6, human genomic DNA was cut with Bgl 2, Pvu 2 and Bgl 1, as described above. The sizes of the restriction fragments that included the target sequence for probes 5 and 6 were 5176, 3843 and 1510 base pairs, respectively, when analyzed on 1.0% agarose gels.

The restriction fragments of human genomic DNA were transferred to nitrocellulose filter paper or to nylon membranes according to the method of E. M. Southern (1975).

b) Analysis Of Human Genomic DNA With Probe 1

DNA impregnated nylon membranes were treated as described in parts b) through d) of Example 3 except that only the first two washes of part b) were employed and color development (part d) was allowed to proceed for only 90 minutes.

c) Analysis Of Human Genomic DNA With Probes 5 And 6

DNA impregnated nitrocellulose filters were bathed in a mixture containg 100 µg of freshly denatured salmon sperm DNA and 10 ml of plaque screen buffer (2 g polyvinylpyrolidone, 2 g ficoll-400, 2 g bovine serum albumin, 58 g sodium chloride, 1 g sodium pyrophosphate, 10 g sodium dodecyl sulfate (SDS) and

950 ml distilled water), at 50°C for 30 minutes. Hybridization was carried out using 40 ng of either probe in 5 ml of buffer at 50°C overnight.

The hybridized filters were washed under high stringency conditions as follows:

a) each filter was washed separately in 100 ml plaque screen buffer at 50°C for 15 minutes

b) the filters were combined and a second wash was done with 0.1% SDS in 3x SSC at 50°C for 15 minutes.

c) the filters were then washed in two changes of a mixture of 1.0 % SDS and 3.2 M trimethyl ammonium chloride at 60°C for 20 minutes

d) the final wash was with 0.1% SDS and 3x SSC at 60°C for 15 minutes.

Filter blocking and visualization were carried out as described in parts c) and d) of example 3, except that color development was terminated after 2 hours.

d) Results

Probes 1 and 5, which are complementary to regions of the tPA gene, detected their respective targets. Discrete bands corresponding to the target DNA bands were clearly visible on the DNA impregnated filters. Probe 1 detected restriction fragments of 5803, 1570 and 3390 base pairs on the Bgl 2, Eco RI and Hind 3 digested DNA, respectively. Probe 5 detected restriction fragments of 5176, 3843 and 1570 base pairs in the Bgl 2, Pvu 2 and Bgl 1 digested DNA, respectively. In contrast probe 6, which was mismatched to the target DNA at one position did not hybridize to any bands.

These results demonstrate that the labeled probes of this invention can discriminate between targets that differ in only a single nucleotide. It

is also significant that probe 5, which was only 24 nucleotides in length and contained only 37% guanine and cytosine was effective.

Example 5
DETECTION OF ANTIGEN ANTIBODY COMPLEXES

In another embodiment of this invention, the biotinylated peptide label is attached to antibodies specific to proteins to be detected. Alternatively, the label may be attached to an antigen, and an antibody specific thereto may be detected. In yet another embodiment, one labeled antibody may be used to detect the presence of a second antibody. And in another embodiment, the label may be attached to a protein or polypeptide which specifically binds to a receptor, the receptor not being either an antigen or antibody, as for example, a cell surface receptor for a hormone, in order to detect the presence of such receptors. In the interest of simplifying the following discusion, the term "specific binding reagent" will be used to describe that moiety to which the label is attached, it being understood that the specific binding reagent may be any proteinaceous substance which binds specifically to a substance whose presence is to be determined, that substance being defined as the target of the specific binding reagent.

Because the label preferably carries multiple bridging moieties, it is possible to attach multiple signalling moieties to each antibody molecule by way of single point of attachment between the label and the antibody.[a] In contrast, most existing methods of antibody labeling require

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[a] Any signalling moiety may be employed. Examples of useful signalling moieties include enzymes, fluorescent compounds, radioactive isotopes, and coumarin.

attachment of multiple signalling moieties to each antibody molecule with corresponding risk of alterating specificity, or a "sandwich" employing second antibodies (See, e.g., U.S. patent 4,376,110) to achieve comparable signal intensity.

Because antibody molecules have both free amino groups (ε-amino groups of lysine and N-terminal amino groups) and cysteine residues, the chemistry for attaching the label to the antibody is preferably different from other embodiments of this invention. In the presently preferred embodiment, the peptide is first biotinylated at reactive ε-amino, α-amino and δ-amino groups (if present). A linkage group is formed by reacting an amino and sulfhydryl reactive hetero bifunctional reagent, preferably succinimyidyl 4-[N-maleimidoethyl] cyclohexane-1-carboxylate ("SMCC") with a sulfhydryl group, preferably at a cysteine residue of the peptide. The cysteine with which the amino and sulfhydryl reactive hetero bifunctional reagent reacts may occur at any point in the peptide, including its amino or carboxy termini. Other amino and sulfhydryl reactive hetero bifunctional reagents, such as meta-maleimiobenzoyl-N-hyrdosuccinimide ester, N-succinimydyl [4-iodoacetyl] aminobenzoate, N-succinimydyl [4-bromoacetyl] and -succinimydyl 3-[2-pyridylthio] propionate are also within the scope of this emodiment. Mixing the derivitized peptide and the specific binding reagent then effects covalent attachment of the derivitized label to the specific binding reagent by reaction of the amino reactive function of the amino and sulfhydryl reactive hetero bifunctional reagent with free amino groups of the specific binding reagent.

Alternatively, it is possible to attach a peptide label which includes at least one cysteine residue to a specific binding reagent that also

includes at least one cysteine residue without the use of a linkage group. This is because upon mixing, cysteine residues will react with one another to form disulfide linkages between them. This reaction has realtively slow kinetics as compared with the reaction between SMCC and cysteine residues. The reaction is carried out overnight with gentle rocking at 4°C.

a) Biotinylation Of Peptide Label

Five mg. of the peptide label of Example 2 a) was was taken up in 500 µl of 0.2 M $NaHCO_3$ at pH 8.2. To this solution was added 10 mg of biotin-N-hydrosuccinimide ester in 200 µl of acetonitrile, in order to achieve a molar excess of biotin. An aliquot of 100µl of water was added to clear turbidity. The reaction was allowed to proceed for four hours at room temperature. Next, 20 mg of dithiothreitol was added to provide reducing conditions; the reaction was allowed to proceed for 16 hours. The resulting solution was desalted on a 5 ml G25 column in phosphate buffered saline ("PBS") (0.137 M NaCl, 2.7 mM KCl, 7.8 mM $Na_2HPO_4 \cdot 7H_2O$ and 1.47 mM KH_2PO_4). Fractions containing the biotinylated peptide were pooled. The derivitized peptide was further purified by HPLC using a C_{18} column. The derivitized peptide was eluted from the C_{18} column with a gradient of from 0 to 100% acetonitrile in 0.1% trifluoroacetic acid over 25 minutes. Fractions containing the derivitized peptide were collected and lyophylized.

b) Attachment Of Peptide Label To Antibody

The derivitized peptide label from part a) of this example was resuspended in 300 µl of PBS at pH 7.2. Two mg of SMCC in 50 µl of acetonitrile was added to this solution. The reaction was allowed to proceed for 1 hour. The reaction mixture was then

purified by HPLC as described in part a) of this example. The derivitized peptide bound to the SMCC linkage group ("peptide") was collected and quantitated by measuring absorbance at 2710 Å.

One mg of a monoclonal antibody to lipocortin-1 was combined with 0.3 or 0. 45 µg of peptide in 300 µl of PBS at pH 7.2. The reaction was allowed to proceed for 16 hours with gentle rocking at 4°C. At the end of this period, the reaction mixture was dialyzed against 1x PBS (to which had been added 0.2% sodium azide) at 4°C with two changes of buffer. Following dialysis, the volume of the reaction mixture was increased to 1 ml with PBS and the mixture was stored cold.

c) Results

Purified lipocortin-1 was mixed with electrophoresis running buffer (50 mM Tris HCl at pH 6.8, 12.5% glycerol, 2% SDS and 2% mercaptoethanol), heated at 65°C for 10 minutes and analyzed by SDS PAGE [Laemmli, Nature, 227, pp. 60-85 (1970)]. Individual lanes contained 1, 0.1, 0.01, and 0.001 µg of lipocortin-1. After electrophoresis, proteins were transferred to nitrocellulose according to the method of Towbin et al., Proc. Nat. Acad. Sci U.S.A., 76, pp. 4350-54 (1979).

The nitrocellulose filters were wetted in a mixture of 10 mM Tris HCl and 150 mM NaCl ("Tris buffer") at pH 7.5 for 10 minutes. Non-specific binding sites on the filters were blocked by treating the filters with a solution of 3% bovine serum albumin ("BSA") in Tris buffer for 30 minutes at room temperature. The filters were rinsed in Tris buffer and then incubated individually, with gentle agitation, for 1 hour in 10 ml of a solution of Tris buffer containing 10 µg of antibody labeled according to parts a) and b) of this Example.

Antibody treated filters were extensively washed in about 200 ml of Tris buffer, using 3 changes of buffer for about 10 minutes per change. The filters were then incubated in 10 ml of Tris buffer containing 1.0 µg/ml of streptavidin-alkaline phosphatase conjugate (Example 3 e)) for 10 minutes. Next, the filters were washed in two changes of 150 ml Tris buffer for 10 minutes per change. For color development, the filters were incubated in a mixture of 100 mM Tris HCl, 0.45 M NaCl, and 50 mg $MgCl_2$ at pH 9.5 containing 0.33 mg/ml nitroblue tetrazolium ("NBT") and 0.165 mg/ml 5-bromo-4-chloro-3-indolylphosphate ("BCIP") in the dark for 2 to 3 hours. The color development was stopped by washing the filters in water. Filters treated with 0.30 µg and 0.45 µg of labeled peptide showed reaction product in the lanes loaded with as little as 10 ng of lipocortin-1.

Example 6

USE OF CHEMILUMINESCENT SUBSTRATES IN NON-ISOTOPICALLY LABELED OLIGONUCLEOTIDE HYBRIDIZATION ASSAY

In yet another embodiment of this invention, a substrate for alkaline phosphatase that yields a chemiluminescent product is employed in place of nitroblue tetrazolium ("NBT") and 5-bromo-4-chloro-3-indolylphosphate ("BCIP") substrate of examples 3 and 5. In one embodiment a dioxetane having the formula:

is used as a substrate for alkaline phosphatase. In this embodiment, T is a cycloalkyl or polycycloalkyl group bonded to the 4 membered ring by a spiro linkage; V is H or an enzyme cleavable group; Y is a fluorescent chromophore; X is H, alkyl, aryl, aralkyl; alkaryl, heteroalkyl, heteroaryl, cycloalkyl, cycloheteroalkyl, or enzyme cleavable group, and Z is H or an enzyme cleavable group, provided that at least one of V, X, Y or Z must be an enzyme cleavable group, so that the enzyme cleaves the enzyme-cleavable group from the dioxetane to form an unstable negatively charged species that further decomposes releasing the chemical bonding energy of dioxetane as light. More preferably this substrate is 3-(2'-spiroadamantane)-4-methoxy-4-(3'-phosphoryloxy)phenyl-1,2-dioxetane ("AMPPD"). High energy chemiluminescent substrates such as AMPPD permit faster analysis and more sensitive detection of binding between probe and analyte. Cleavage of AMPPD by alkaline phosphatase yields a chemiluminescent species which can be detected by optical instrumentation. See EPO applications 254,051 and WP 88/00695. Although the results presented in this example are based on use of AMPPD with a DNA probe, the methodology presented may be also employed in connection with the specific binding reagent disclosed in Example 5. Additionally, it should be understood that any other combination of enzyme and substrate compound which produces a chemiluminescent species upon reaction between the enzyme and substrate is within the scope of this embodiment.

a) Preparation of Samples

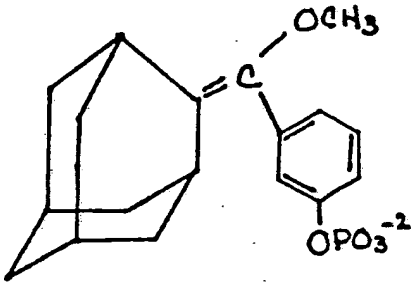
Southern blots of human genomic DNA were prepared according to the procedure of Example 4a). Aliquots of 20 µg of DNA were digested with the restriction endonucleases Bg12, EcoR1 and Hind3 to

generate fragments of 5803, 1570 and 3390 base pairs respectively as in Example 4a). The digested DNA was subjected to electrophoresis on a 1% agarose gel. After denaturation and neutralization, the DNA fragments were transferred to nylon membranes also as described in Example 4a).

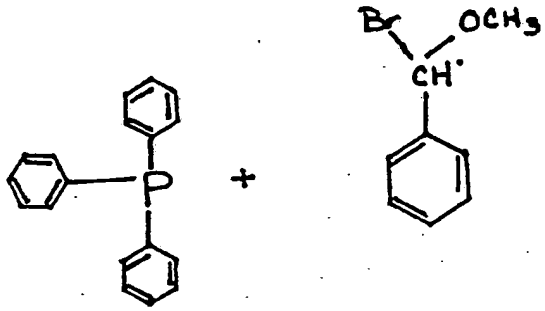
DNA probes identical to Probe 1 of Example 4 were hybridized to the genomic DNA according to the protocol described in Example 3b) and c), except that the probes were used at a concentration of 8 ng/ml of the hybridization solution. The filters were then washed and blocked, also according to the protocol disclosed in Examples 3c) and d).

b) Preparation Of Substrate

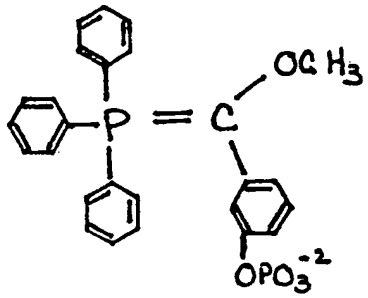
The alkaline phosphatase substrate AMPPD may be produced by synthesizing the olefinic intermediate A:



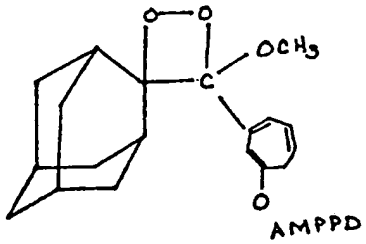
This intermediate is synthesized by the reaction of spiroadamantanone and a phosphorous ylide. The phosphorous ylide is formed by the reaction of triphenyl phosphine and the brominated benzene derivative:



in the presence of the strong base, n-butyl lithium, at -78°C in tetrahydrofurane to yield a phosphorous ylide;



This ylide, upon reaction with spiroadamantanone yields the olefinic intermediate A.



Irradiation of the olefinic intermediate A in the presence of singlet oxygen yields AMPPD.

b) Detection

The performance of NBT/BCIP and AMPPD as substrates for alkaline phosphatase was evaluated by comparing the results obtained with these substrates on otherwise identically treated filters. One filter was treated as described in Example 3e). A second filter, after blocking with 3% w/v BSA in a solution of 0.1M Tris HCl and 0.45 NaCl at pH 7.5, was incubated with streptavidin-alkaline phosphatase solution (0.1 mg/ml in Tris buffer as described in Example 3e)) for 15 minutes at room temperature. Following incubation, the filter was washed in two changes of 150 ml of a solution of 0.1 M Tris HCl and 0.45 NaCl for 15 minutes at room temperature.

The membranes were washed in a solution consisting of 0.1% BSA, 5 mM $NaHCO_3$, and 1 mM $MgCl_2$ at pH 9.5. After washing, the membrane was saturated

with 100 µl of 1.6 mM AMPPD in 0.1% BSA, 5 mM $NaHCO_3$, and 1 mM $MgCl_2$ at pH 9.5. The membrane was sealed in a plastic pouch and placed in a camera luminometer where light emission was imaged on Polaroid Instant Film (ASA 20,000). Membranes treated with NBT/BCIP substrates produced detectable signals in 30 minutes, whereas the AMPPD produced detectable signals within 2 minutes. Additionally, the chemiluminescent product permits a more sensitive assay than with conventional NBT/BCIP technology. It should be understood that other detectors of chemiluminescence may be used in place of the photographic film of this example. Such detectors include video equipment and solid state photon detectors.

EXAMPLE 7

NON-RADIOACTIVE DETECTION OF TARGET GENES IN HUMAN GENOMIC DNA USING PCR TECHNOLOGY

Polymerase Chain Reaction technology ("PCR") is being used increasingly in various aspects of molecular biology, including medical diagnostics. The methodology provides for detection of target sequences from very small amounts of DNA isolated from cells or tissues. This embodiment discloses a method for rapid and sensitive detection of target sequences employing PCR. Also within the scope of this embodiment is a rapid method for preparation of DNA samples from blood for PCR.

Briefly, PCR is a method for amplifying a target nucleotide sequence by causing a heat stable DNA polymerase and oligonucleotide primers to generate DNA extension products from nucleotide monomers, beginning from a site that at which primers bind to complementary polynucleotide strand templates. The primers are selected so that they bracket the sequence to be amplified. Following generation of fragments from the template strand, elevation of the

temperature of the reaction medium causes the dissociation of the template strands, the heat stable DNA polymerase and the extension fragments without inactivating the DNA polymerase enzyme. In a preferred embodiment, the heat stable DNA polymerase is derived from Thermus aquaticus. Each extension product can then serve as a template for another round of synthesis, thus amplifying the amount of sequence sought exponentially. See generally EPO Application 258,017.

The probe labelling and sample preparation methodology of this invention can be combined with PCR to provide a powerful technique for detection of target sequences. In this Example these embodiments are employed in the detection of the tPA gene in human genomic DNA.

a) DNA Isolation From Cells Suspended In Fluids For PCR

An aliquot of 50 µl of human blood was mixed with 200 µl of a cold solution of 0.15 M NaCl and 50 mM Tris HCl at pH 7.5. The solution was mixed at low speed using a laboratory vortex mixer. The cells were then spun down by centrifugation at about 5,000 rpm for 1 minute. After discarding the supernatant, the cells were washed by resuspending the cells in the above buffer and repeating the mixing and centrifugation steps.

The pelleted cells were then treated with 100 µl of NP40 in 1x PBS (any other nonionic detergent may be employed in place of NP40) at 4°C. The mixture was vortexed briefly, and the nuclei were spun down by centrifugation at 5,000 rpm for 1 minute. The supernatant was gently removed by pipetting. The nuclei were washed by adding 100 µl of a cold solution of 0.15 M NaCl and 50mM Tris HCl at pH 7.5. The liquid was again gently removed by pipetting.

Next, 10 µl of 1% sodium deoxycholate (any negatively charged detergent may be used in place of sodium deoxychiolate[a]) was added to the nuclear pellet. The mixture was vortexed and centrifuged as before. The solution was allowed to stand for 15 minutes at room temperature. The solution was then boiled for 5 minutes to solubilize the DNA, and again vortexed and centrifuged. An aliquot of 10 µl of double distilled water was added to the DNA solution, and 10 µl of the resulting solution was used for the remainder of this example. The resulting DNA solution was diluted to 500 µl with a mixture of 10 mM Tris HCl and 1 mM EDTA (ethylene diamine tetraacetic acid) at pH 7.5, vortexed and centrifuged as before. 50 µl of this solution was used as a source of human DNA for PCR. It will also be apparent that although this Example employs small amounts of human blood as a sample, this technique may be applied to other cell suspensions, as well as to larger or smaller volumes. For example, this technique may also be used to study nucleic acids from cells grown in culture, or to a sample of synovial fluid.

Although it is preferred to employ this method for isolation of DNA in the preparation of samples for PCR, it is also within the scope of this embodiment to employ the method for preparation of DNA for other purposes.

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[a] Sodium dodecylbenzene sulfonate (also known as sodium dodecyl sulfate) at 1% concentrations may be used in place of sodium deoxycholate for nuclear lysis. If so, it must be removed using C_{18} silica-solid support (Whatman), prior to amplification with PCR. Companion experiments done with this detergent yielded results comparable to those with sodium deoxycholate.

b) Amplification Of Target Sequences In Human Genomic DNA

Two primers were synthesized using the methods disclosed in Example 1a). The primers had the sequence:

Primer A: 5' AAA GTG CTG GGA TTA CCA GC 3'

Primer B: 5' TGG CCT CCT AAA GTG CTG GG 3'

Primer A is complementary to the + strand coding for tPA and Primer B is complementary to the corresponding - strand. Use of these primers results in amplification of a 350 base pair DNA fragment.

The PCR reaction was carried out in the following reaction mixture:

10x buffer	10 µl[a]
human DNA	10 µl[b]
double distilled H_2O	53.5 µl
deoxynucleotide triphsophates	16.0 µl[c]

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[a] The PCR reaction buffer consists of 500 mM KCl, 100 mM Tris HCl, 15 mM $MgCl_2$ and 0.1% w/v gelatine at pH 8.3.

[b] Paired experiments were carried out using human DNA prepared according to part a) of this example and with human DNA prepared according to conventional technology. See, e.g. P. E. Devlin et al. "Southern Analysis Of Genomic DNA with Unique and Degenerate Oligonucleotide Probes: A Method for Reducing Degeneracy," DNA, I, pp. 499-507 (1988). When the source of human DNA was human blood, treated according to part a) of this Example, 50 µl of the final solution was used for the PCR reaction mixture. The volume of double distilled H_2O in that reaction mixture was correspondingly reduced by 40 µl to 13.5µl.

[c] A mixture of the 4 deoxynucleotides normally present in human cells (i.e., dATP, dGTP, dCTP and dTTP) each present at a concentration of 200 µM was used.

Primer A	5.0 µl[d]
Primer B	5.0 µl[d]
Taq polymerase	0.5 µl[e]

Each PCR cycle consisted of the following steps:

1. denaturation for 2 minutes at 93°C
2. primer annealing for 2 minutes at 40°C
3. chain extension for 4 minutes at 60°C

These three steps were carried out for a total of 40 cycles in a Perkin-Elmer-Cetus Thermocycler. The initial mass of human genomic DNA in the PCR reaction mixture was 100 ng., 10 ng., 1 ng., and 0.1 ng for each of 4 separate experiments.

c) Detection

After completion of the above steps, 10 µl of the reaction mixture was denatured at 60°C for in 15 µl of a mixture of 0.5 M NaOH and 1.5 M NaCl. After 1 hour, the mixture was neutralized with 200 µl of 0.5 M Tris and 1.5 M NaCl at pH 7.5. Samples were blotted onto nitrocellulose filters using a slot blotting apparatus [Minifold 2 Slot Blotter, Sleicher & Schule].

Filters were hybridized in 10 ml of PBS containing 100 µg/ml of sonicated salmon sperm DNA and 8 ng of a probe with the sequence of probe 6 in Example 4 at 55°C overnight. the flters were washed in the following solution:

5x SSC[f] and 0.1% SDS at 55°C for 10 minutes (2 changes)

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[d] At a concentration of 1 µM

[e] correponds to 2.5 units. The Taq polymerase enzyme was obtained from Perkin Elmer-Cetus; the volume indicated in the text was used from the material supplied.

[f] The formula for 6x SSC is provided in Example 3c).

3x SSC and 0.1% SDS at 50°C for 10 minutes
1x SSC and 0.1% SDS at 50°C for 10 minutes
(2 changes)

After washing the filters, nonspecific binding sites on the filters were blocked according to the procedure of Example 3d). Bound probe was detected according to the method of Example 3e).

Additionally, after completion of the PCR reactions, 10µl from each of the four experiments was subjected to agaraose gel electrophoresis according to method of Example 4 except that a 2% agarose gel was used. Following denaturation and neutralization the DNA was transferred onto nitrocellulose or Gene-screen™ filters also as described in Example 4. The filters were washed, blocked and the bound probe detected also as described in Example 4 using probes with the sequence of probe 6 of that Example.

d) Results

The combination of PCR and probe labeling allowed detection of target sequence in as little as 100 pg of starting genomic DNA following electrophoresis and Southern blotting of the product of the PCR reaction. This corresponds to roughly 30 molecules of DNA in the PCR reaction mixture. We confirmed that the DNA fragment detected was the 350 base pair fragment amplified by PCR by Southern blotting the DNA fragments after separation by agarose gel electrophoresis. The 350 base pair amplified fragment was also detected when the human DNA was prepared according to part a) of this example. In that case a gene was detected in the equivalent of 0.625 µl of human blood.

Example 8

USE OF PROBE LABELING IN ANTISENSE THERAPY

Antisense therapy refers to the administration of exogenous polynucleotides which bind to target polynucleotides located within cells. The term "antisense" refers to the fact that such oligonucleotides are complementary to their intracellular targets. Useful oligonucleotide sequences interfere with or modulate the processes of growth and replication. It is believed that this methodology will permit development of novel antiviral and antineoplastic pharmaceutical agents. See "Antisense Molecular Biology and 'S-oligos'", Synthesis, 1, pp. 1-5 (Synthecell, October 1988) for a general review.

A major obstacle in the development of useful antisense therapeutic agents has been the intracellular location of their targets. Id. See also, LeMaitre, et al. "Specific Antiviral Activity Of A Poly(L-lysine))-Conjugated Oligodeoxyribonucleotide Sequence Complementary to Vesicular Stomatitis Virus N Protein mRNA Initiation Site", Proc. Nat. Acad. Sci. U.S.A., 84, pp. 648-52, 648 (1987). Accordingly, it is desirable to develop a method for intracellular delivery of such agents.

In one embodiment of the present invention, antisense therapeutic agents ("antisense agents") are bound to a peptide that is ingested by cells ("peptidyl antisense agent"). Examples of useful peptides include peptide hormones, antigens or antibodies, and peptide toxins. By choosing a peptide that is selectively taken up by a group of cells or a tissue that is to be exposed to the antisense agent, specific delivery of the antisense agent may be effected.

The antisense agent is covalently bound to a peptide, that is known to be ingested by cells, according to the method of Examples 1 and 2. Briefly,

the 5'-OH group of the antisense agent is activated by aminoalkylation. A peptide is covalently attached to the activated antisense agent by an amino and sulfhydryl reactive hetero bifunctional reagent. The latter is bound to a cysteine residue present in the peptide.

Upon exposure of cells to the antisense agent bound to a peptide, the peptidyl antisense agents are endocytosed, and the antisense agent binds to target polynucleotides within the cells, thus exerting its therapeutic effect.

CLAIMS

We claim:

1. A labeled probe comprising a polynucleotide complementary to a target polynucleotide, a label including a sulfhydryl group, and a linkage group, the linkage group formed by the reaction of an amino and sulfhydryl reactive heterobifunctional reagent with a sulfhydryl group of the label, the reaction resulting in the oxidation of the sulfhydryl group.

2. The labeled probe of claim 1, wherein the label comprises a polypeptide including a cysteine residue.

3. The labeled probe of claim 1, wherein at least one signalling moiety is attached to the label.

4. The labeled probe of claim 3, wherein at least one signalling moiety is attached to the label by way of at least one bridging moiety.

5. The labeled probe of claim 4, wherein the bridging moiety is selected from the group consisting of biotin, iminobiotin, avidin, streptavidin and an antibody to the label.

6. The labeled probe of claim 3, 4 or 5, wherein the signalling moiety is selected from the group consisting of radioactive isotopes, enzymes coumarin, and fluorescent compounds.

7. The labeled probe of claim 1, 2, 3 or 4 wherein the linkage group is attached to an amino group at the 5' terminus of probe.

8. The labeled probe of claim 1, 2, 3 or 4 wherein the linkage group is attached to a base.

9. A method for labeling a probe molecule comprising the steps of:

a) selecting a probe molecule comprising a sequence of bases complementary to a target sequence;

b) aminating a 5'-OH group of the probe molecule;

c) attaching a linkage group to the aminated probe molecule with an amino and sulfhydryl reactive hetero bifunctional reagent; and

d) binding a label to the linkage group of the probe molecule.

10. The method for labeling a probe molecule of claim 9, wherein the step of aminating further comprises the steps of:

a) reacting the probe molecule with an azolide; and

b) derivatizing the probe molecule of step a) above with a diamine.

11. The method for labeling a probe molecule of claim 10, wherein the azolide is carbonyldiimidazole.

12. The method for labeling a probe molecule of claim 10, wherein the diamine is hexamethylinediamine.

13. The method for labeling a probe molecule of claim 9, wherein the amino and sulfhydryl reactive hetero bifunctional reagent is

selected from the group consisting of 4-[N-maleimidomethyl] cyclohexane-1-carboxylate, meta-maleimidobenzoyl-N-hydrosuccinimide ester, N-succinimidyl [4-iodoacetyl] aminobenzoate, N-succinimidyl [4-bromoacetyl] and N-succinimidyl 3-[2-pyridylthio] propionate.

14. The method for labeling a probe molecule of claim 9, wherein the step of binding further comprises the step of oxidizing a sulfhydryl group on the label with the linkage group.

15. The method for labeling a probe molecule of claim 9, wherein the step of binding further comprises the step of attaching at least one signalling moiety to the label.

16. A method for labeling a probe molecule comprising the steps of:

a) selecting a probe molecule comprising a sequence of bases complementary to a target sequence;

b) covalently binding a linkage group bearing a reactive amino group to at least one base of the probe molecule;

c) attaching an amino and sulfhydryl reactive heterobifunctional reagent to the amino group of the linkage group to form a second linkage group; and

d) binding a label to the second linkage group by the oxidation of a sulfhydryl group.

17. The method for labeling a probe molecule of claim 16, wherein the amino and sulfhydryl reactive heterobifunctional reagent is selected from the group consisting of 4-[N-maleimidomethyl]

cyclohexane-1-carboxylate, meta-maleimidobenzoyl-N-hydrosuccinimide ester, N-succinimidyl [4-iodoacetyl] aminobenzoate, N-succinimidyl [4-bromoacetyl] and N-succinimidyl 3-[2-pyridylthio] propionate.

18. The method for labeling a probe molecule of claim 16, wherein the step of binding further comprises the step of attaching at least one signalling moiety to the label.

19. The method for labeling a probe molecule of claim 18, wherein the step of attaching further comprises the step of attaching the at least one signalling moiety to the label by at least one bridging moiety.

20. A method for detecting a target in genomic DNA comprising the steps of:

a) adding a probe labeled according to the method of claim 9, 10, 11, 12, 13, 14 or 15 to a sample of genomic DNA;

b) allowing the labeled probe to hybridize to target molecules;

c) removing labeled probe molecules which are not bound to target molecules;

d) detecting a signal from at least one signalling moiety of the labeled probe molecules.

21. A method for detecting a target in genomic DNA comprising the steps of:

a) adding a probe labeled according to the method of claim 16, 17, 18, or 19 to a sample of genomic DNA;

b) allowing the labeled probe to hybridize to target molecules;

c) removing labeled probe molecules which are not bound to target molecules;

d) detecting a signal from at least one signalling moiety of the labeled probe molecules.

22. A labeled specific binding reagent comprising a polypeptide that binds specifically to an analyte, and a label including a sulfhydryl group attached to the polypeptide by reaction of an amino and sulfhydryl reactive hetero bifunctional reagent with the label and the specific binding agent resulting in oxidation of the sulfhydryl group.

23. The labeled specific binding reagent of claim 22, wherein the label comprises a polypeptide including a cysteine residue.

24. The labeled specific binding reagent of claim 22, wherein the amino and sylfhydryl reactive hetero bifunctional reagent is selected from the group consisting of succinimyidyl 4-[N-maleimidomethyl] cyclohexane-1-carboxylate, metamaleimidobenzoyl-N-hyrdosuccinimide ester, N-succinimydyl [4-iodoacetyl] aminobenzoate, n-succinimydyl [4-bromoacetyl] and -succinimydyl 3-[2-pyridylthio] propionate.

25. The labeled specific binding reagent of claim 22, wherein at least one signalling moiety is attached to the label.

26. The labeled specific binding reagent of claim 48, wherein the signalling moiety is selected from the group consisting of enzymes, radioactive isotopes, coumarin and fluorescent compounds.

27. The labeled specific binding reagent of claim 25, wherein at least one signalling moiety

is attached to the label by way of at least one bridging moiety.

28. The labeled specific binding reagent of claim 27, wherein the bridging moiety is selected from the group consisting of biotin, iminobiotin, avidin, and streptavidin.

29. A method for labeling a specific binding reagent comprising the steps of:

a) selecting a specific binding reagent comprising a polypeptide that is specific to an analyte;

b) attaching at least one bridging moiety to at least one amino group on a label, the label including a sulfhydryl group;

c) derivitizing the label by reacting an amino and sulfhydryl reactive hetero bifunctional reagent with the label resulting in oxidation of the sulfhydryl group;

d) binding the label to the specific binding reagent.

30. The method for labeling a specific binding reagent of claim 29, wherein the step of attaching further comprises reacting amino groups of the label with bridging moieties selected from the group comprising biotin, and iminiobiotin, under reducing conditions.

31. The method for labelling a specific binding reagent of claim 29, wherein the amino and sulfhydryl reactive heterobifunctional reagent is selected from the group consisting of succinimyidyl 4-[N-maleimidomethyl] cyclohexane-1-carboxylate, metamaleimidobenzoyl-N-hyrdosuccinimide ester, N-succinimydyl [4-iodoacetyl] aminobenzoate,

n-succinimydyl [4-bromoacetyl] and -succinimydyl 3-[2-pyridylthio] propionate.

32. The method for labeling a specific binding reagent of claim 29, wherein the step of binding further comprises the step of attaching at least one signalling moiety to the label.

33. A method for labeling a specific binding reagent that includes at least one sulfhydryl group, comprising the steps of:

a) selecting a peptide label comprising at least one amino acid residue that contains a sulfhydryl group;

b) mixing the specific binding reagent and peptide label under conditions that allow formation of disulfide bonds between cysteine groups present on the peptide label and the specific binding reagent.

34. A method for detecting the presence of a target in a mixture containing the target comprising the steps of:

a) exposing the mixture to a labeled probe, the labeled probe comprising a first polynucleotide complementary to a second target polynucleotide, a label including a sulfhydryl group and a linkage group formed by the reaction of an amino and sulfhydryl reactive hetero bifunctional reagent with the label, the reaction resulting in the oxidation of the sulfhydryl group, the linkage group being attached to the 5' terminus of the probe.

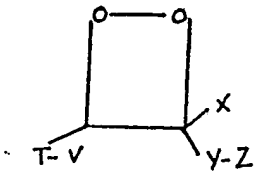
b) removing labeled probe that is not bound to the target;

c) reacting labeled probe that is bound to the target with a substrate that upon reaction

with the labeled probe yields a chemiluminescent product;

d) detecting the presence of the chemiluminescent product.

35. The method for detecting the presence of a target of claim 34, wherein the substrate is a dioxetane of the formula:



where T is a cycloalkyl or polycycloalkyl group bonded to the 4 membered ring by a spiro linkage; V is H or an enzyme cleavable group; Y is a fluorescent chromophore; X is H, alkyl, aryl, aralkyl; alkaryl, heteroalkyl, heteroaryl, cycloalkyl, cycloheteroalkyl, or enzyme cleavable group, and Z is H or an enzyme cleavable group, provided that at least one of V, X, Y or Z must be an enzyme cleavable group, so that the enzyme cleave the enzyme-cleavable group from the dioxetane to form an unstable negatively charged species that further decomposes releasing the chemical bonding energy of dioxetane as light.

36. The method for detecting the presence of a target of claim 35, wherein the dioxetane is AMPPD.

37. The method for detecting the presence of a target in a mixture containing the target of claim 34, wherein the amino and sulfhydryl reactive hetero bifunctional reagent is selected from the group consisting of succinimyidyl 4-[N-maleimidomethyl] cyclohexane-1-carboxylate, metamaleimidobenzoyl-N-hydrosuccinimide ester, N-succimydyl [4-iodoacetyl]

aminobenzoate, n-succinimydyl [4-bromoacetyl] and -succinimydyl 3-[2-pyridylthio] propionate.

38. A method for detecting the presence of a target of a specific binding reagent in a mixure containing the target of the specific binding reagent comprising the steps of:

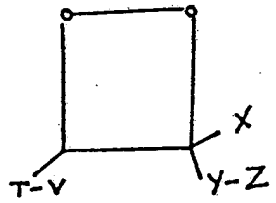
a) exposing the mixture to a specific binding reagent labeled according to the method of claim 29, 30, 31 or 32;

b) removing labeled specific binding reagent that is not bound to the target of the specific binding reagent;

c) reacting bound labeled specific binding reagent with a substrate that upon reaction yields a chemiluminescent product;

d) detecting the chemiluminescent product.

39. The method for detecting the presence of a target of a specific binding reagent of claim 38, wherein the substrate is a dioxetane of the formula:



where T is a cycloalkyl or polycycloalkyl group bonded to the 4 membered ring by a spiro linkage; V is H or an enzyme cleavable group; Y is a fluorescent chromophore; X is H, alkyl, aryl, aralkyl; alkaryl, heteroalkyl, heteroaryl, cycloalkyl, cycloheteroalkyl, or enzyme cleavable group, and Z is H or an enzyme cleavable group, provided that at least one of V, X, Y or Z must be an enzyme cleavable group, so that the enzyme cleave the enzyme-cleavable group from the deoxetane to form a negatively charged substituent

bonded to the dioxetane, the negatively charged substituent causing the dioxetane to decompose to form a luminescent substance that includes group Y of said dioxetane.

40. The method for detecting the presence of a target of a specific binding reagent of claim 39, wherein the dioxetane is AMPPD.

41. A method for isolating DNA from a suspension of cells comprising the steps of:

a) isolating cells from a sample;

b) treating washed and isolated cells with a nonionic detergent;

c) separating nuclei from the detergent treated cells; and

d) extracting DNA from the isolated nuclei.

42. The method for isolating DNA from a suspension of cells of claim 41, wherein the nonionic detergent is NP40.

43. The method for isolating DNA from a suspension of cells of claim 41, wherein the step of extracting further comprises the steps of:

a) adding an aliquot of negatively charged detergent to the isolated nuclei; and

b) solubilizing the DNA from the isolated nuclei.

44. The method for isolating DNA from a suspension of cells of claim 43, wherein the negatively charged detergent is selected from the group consisting of sodium dodecylbenzene sulfonate and sodium deoxycholate.

45. A method for determining the presence of a target in a mixture comprising the steps of:

a) isolating an aliquot of nucleic acid;

b) amplifying a portion of the nucleic acid which may include the target;

c) detecting the presence of the target with a labeled probe, the method of labeling the probe comprising the steps of:

i) activating the 5'-OH terminus of the probe by amino alkylation;

ii) attaching at least one signalling moiety to a label comprising a sulfhydryl group and at least one amino group;

iii) linking the label to the probe by reacting the probe and label with an amino and sulfhydryl reactive heterobifunctional reagent, the reaction resulting in the oxidation of the sulfhydryl group.

46. A method of delivering an antisense therapeutic agent to cells by attaching an antisense therapeutic agent to a polypeptide ingested by cells comprising the steps of:

a) aminating the 5'-OH group of the antisense therapeutic agent;

b) attaching a linkage group comprising an amino and sulfhydryl reactive hetero bifunctional reagent to the aminated antisense therapeutic agent;

c) binding the linkage group to the polypeptide ingested by cells.

47. The method for delivering an antisense therapeutic agent to cells of claim 46, wherein the step of aminating further comprises the steps of:

a) reacting the antisense therapeutic agent with an azolide;

b) derivitizing the antisense therapeutic agent of step a) with a diamine;

48. The method for delivering an antisense therapeutic agent of claim 47, wherein the azolide is carbonyl-diimidazole.

49. The method for delivering an antisense therapeutic agent of claim 48, wherein the diamine is hexamethylenediamine.

50. The method for delivering an antisense therapeutic agent of claim 46, wherein the amino and sulfhydryl reactive hetero bifunctional reagent is selected from the group consisting of succinimyidyl 4-[N-maleimidomethyl] cyclohexane-1-carboxylate, metamaleimidobenzoyl-N-hyrdosuccinimide ester, N-succinimydyl [4-iodoacetyl] aminobenzoate, n-succinimydyl [4-bromoacetyl] and -succinimydyl 3-[2-pyridylthio] propionate.

51. The method for delivering an antisense therapeutic agent of claim 46, wherein the polypeptide ingested by cells is selected from the group consisting of peptide hormones, antibodies, antigens and toxins.

FIG. 1

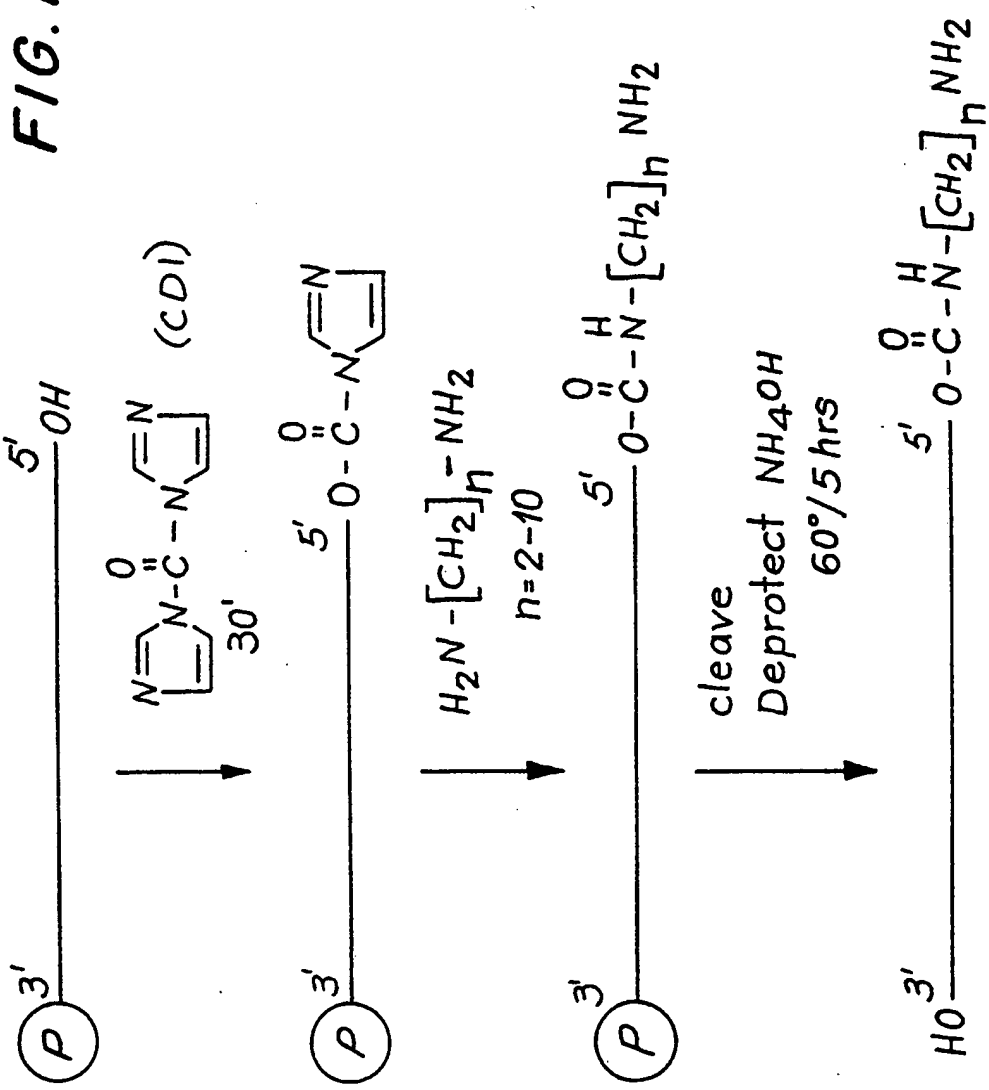
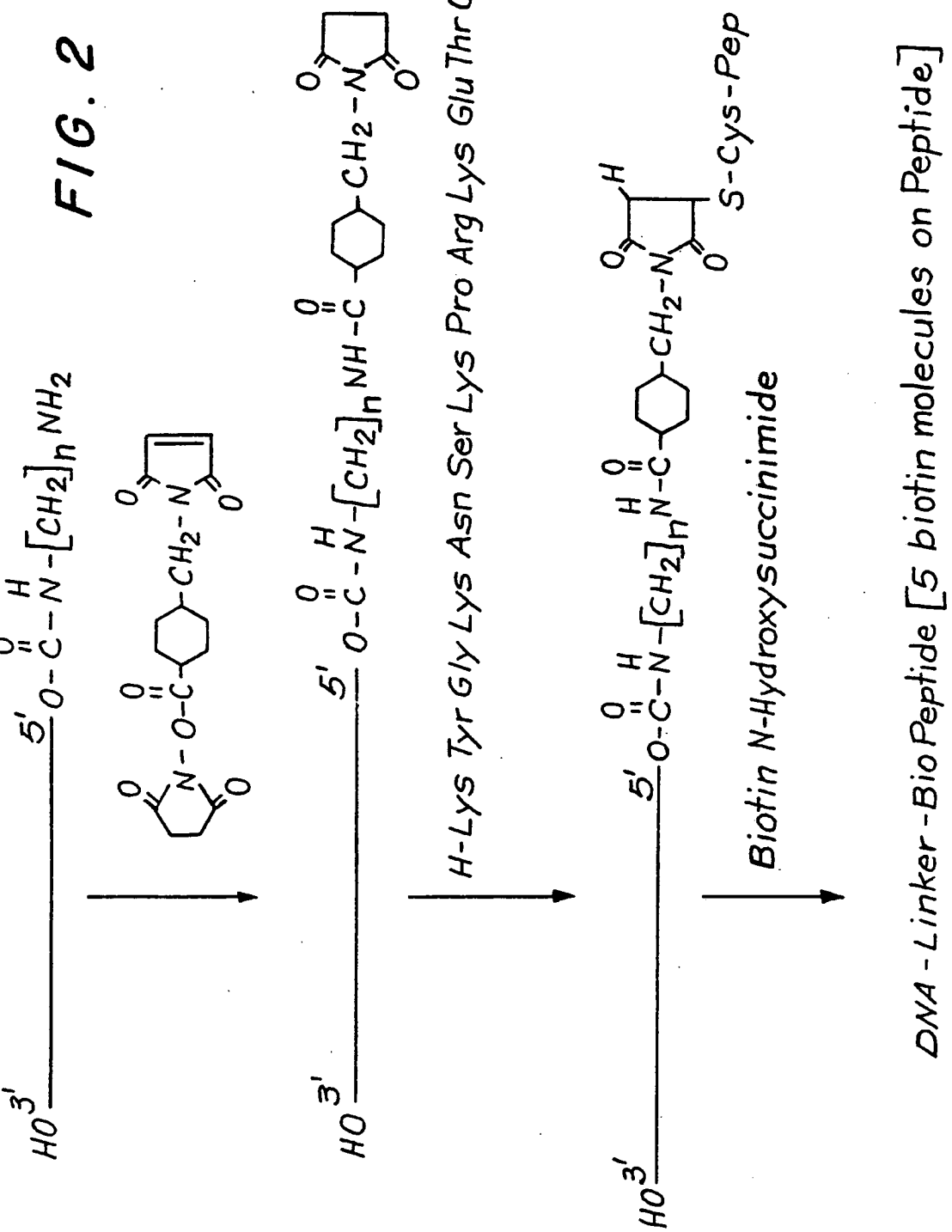

5' OH
3'
P
(CDI)
30'
5' O-C-N
$H_2N-[CH_2]_n-NH_2$
n=2-10
5' O-C-N-$[CH_2]_n$ NH_2
cleave
Deprotect NH_4OH
60°/5 hrs
HO 3'
5' O-C-N-$[CH_2]_n$ NH_2

FIG. 2


H-Lys Tyr Gly Lys Asn Ser Lys Pro Arg Lys Glu Thr Cys-SH
Biotin N-Hydroxysuccinimide
S-Cys-Pep
DNA - Linker - Bio Peptide [5 biotin molecules on Peptide]

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 89/02363

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: C 12 Q 1/68

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC5	C 12 Q

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	Chemical Abstracts, volume 83, 1975, (Columbus, Ohio, US), M. Perrin et al.: "Fluorescent labeling of Escherichia coli ribosomal sulf-hydryl groups", see page 150, abstract 189552j, & Eur. J. Biochem. 1975, 57(2), 319-24 --	1,9,16,29, 33,34,38
X	Biological Abstracts, volume 87, no. 2, 1989, (Philadelphia, PA, US), O.S. Bashchuk et al.: "Synthesis of oligonucleotide derivatives bearing amino and sulfhydryl groups on polymer support: Introduction of spin, fluorescent and other labels", see page AB-47, abstract 12795, & Bioorg. Khim. 14(5): 606-614, 1988 --	1,9,16,29, 33,34,38
X	Chemical Abstracts, volume 104, 1986, (Columbus, Ohio, US), B.A. Connolly et al.: "Chemical synthesis of oligonucleotides ./.	1,9,16,29, 33,34,38

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
10th October 1989	08 NOV 1989
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	T.K. WILLIS

International Application No. PCT/US 89/02363

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
	containing a free sulfydryl group and subsequent attachment of thiol specific probes", see page 699, abstract 88937k, & Nucleic Acids Res. 1985, 13(12), 4485-502	
	--	
X	EP, A, 0154884 (MOLECULAR DIAGNOSTICS INC.) 18 September 1985 see page 4, lines 10-35; page 5, lines 30-35; page 6, lines 1-7; page 6, lines 20-30	2-6,10,12, 15,18,19, 22,23,25-28,30,32, 33
	--	
X	EP, A, 0138357 (INTEGRATED GENETICS INC.) 24 April 1985 see claims 1-33; figures 1-4; page 1, line 30 - page 2, line 6	3,4,6,15, 18,19,25-27,32
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X	EP, A, 0142299 (FUJIREBIO INC.) 22 May 1985 see page 4, lines 5-7	1,9,16,29, 33,34,38
	--	
X	EP, A, 0063879 (YALE UNIVERSITY) 3 November 1982 see page 4, lines 5-22; page 22, lines 11-15; page 26, table III; page 33, lines 1-36	5,20,24
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X	DNA, volume 4, no. 4, 1985, Mary Ann Liebert, Inc., Publishers, (New York, N.Y., US), B.C.F. Chu et al.: "Laboratory methods. Detection of specific DNA sequences with short biotin-labeled probes", pages 327-331 see page 327, column 1, lines 16-21; page 327, column 2, line 21 - page 328, column 1, line 17; page 328, figure 1	7,8,12,13, 17,31,37

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 8902363 SA 29621

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 30/10/89
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0154884	18-09-85	US-A- 4748111	31-05-88
		AU-B- 578933	10-11-88
		AU-A- 3943485	19-09-85
		JP-A- 60226900	12-11-85
EP-A- 0138357	24-04-85	US-A- 4626501	02-12-86
		CA-A- 1234061	15-03-88
		JP-A- 60172994	06-09-85
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EP-A- 0063879	03-11-82	AU-B- 560651	16-04-87
		AU-A- 8257382	21-10-82
		CA-A- 1219824	31-03-87
		EP-A- 0329198	23-08-89
		JP-A- 57209297	22-12-82
		JP-A- 63099093	30-04-88
		US-A- 4711955	08-12-87

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